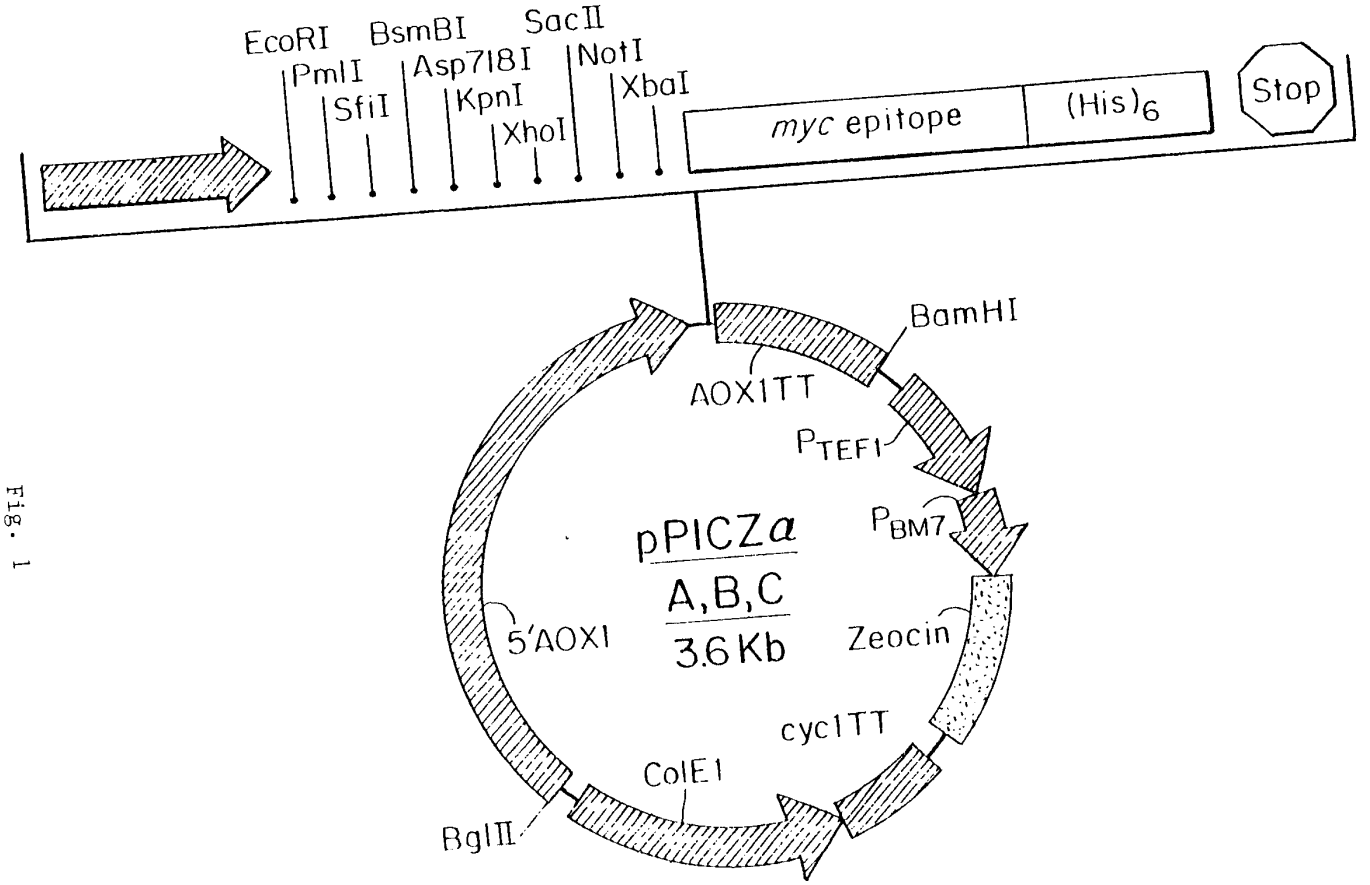
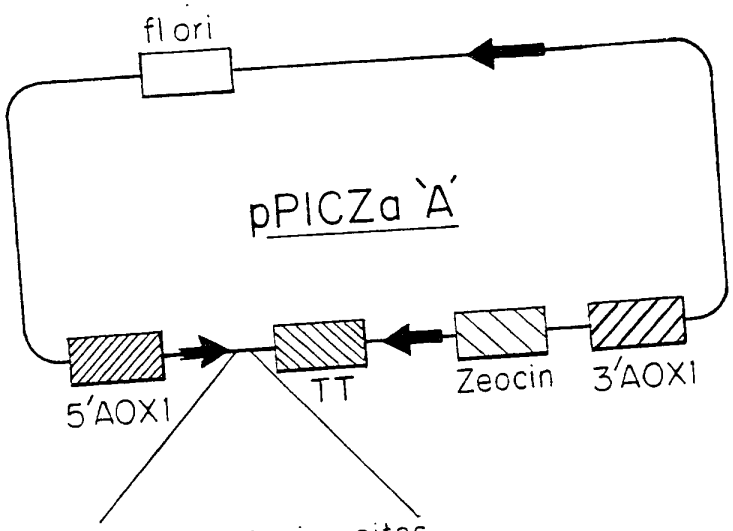

EcoRI
PmlI
SfiI
BsmBI
Asp718I
KpnI
XhoI
SacII
NotI
XbaI
myc epitope
(His)6
Stop
BamHI
AOX1TT
PTEF1
PBM7
pPICZα
A,B,C
3.6Kb
Zeocin
cyc1TT
ColE1
BglII
5'AOX1

Fig. 1



Multiple cloning sites
EcoRI, / PmlI / SfiI / Bsm BI / Asp718I / KpnI / XhoI /
SacII / NotI / XbaI

Digest the vector with EcoRI restriction enzyme

Denature and anneal two complimentary oligo which would incorporate His.Tag motif and NdeI, NheI restriction sites (His. Tag region would be helpful to simplify purification and the presence of NdeI and NheI restriction sites facilitate shuttling of PCR from prokaryotic expression system to yeast expression without going through PCR amplification process)

5' AAT TCC ATC ACC ATC ACC ATC ACC ATA TGG CTA GCA 3'
5' AAT TTG CTA GCC ATA TGG TGA TGG TGA TGG TGA TGG 3'

Modified Pichia expression vector (pPICZ. His. A)

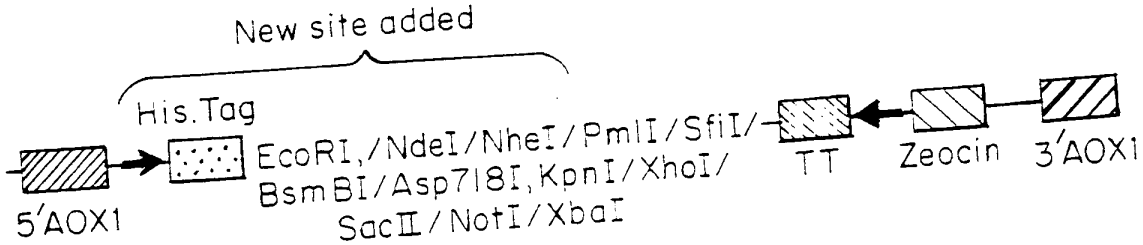


Fig. 2

Flow Chart: Cloning of Endostatin (mouse) into Pichia Expression System

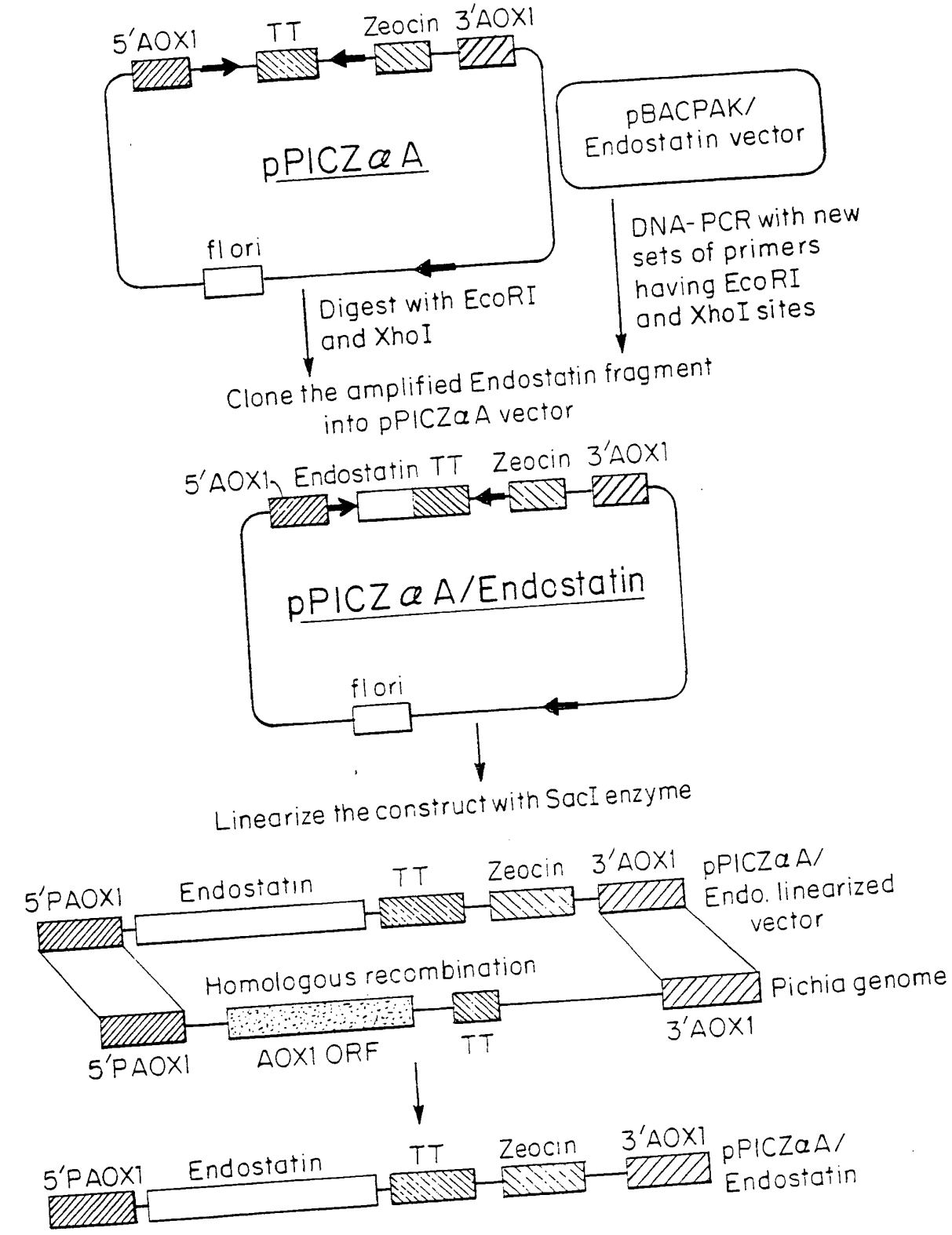


Fig. 3

endo sequence from Collagen XVIII.
Sequence Range: 1-555
Nucleotide 1 = Start for Endostatin and fragments EM1 and EM2.
EM1 fragment ends at nucleotide 525, EM2 fragment ends at
nucleotide 501.

```
     5    10    15    20    25    30    35    40    45
CAT ACT CAT CAG GAC TTT CAG CCA GTG CTC CAC CTG GTG GCA CTG AAC
GTA TGA GTA GTC CTG AAA GTC GGT CAC GAG GTG GAC CAC CGT GAC TTG

 50    55    60    65    70    75    80    85    90    95
ACC CCC CTG TCT GGA GGC ATG CGT GGT ATC CGT GGA GCA GAT TTC CAG
TGG GGG GAC AGA CCT CCG TAC GCA CCA TAG GCA CCT CGT CTA AAG GTC

100   105   110   115   120   125   130   135   140
TGC TTC CAG CAA GCC CGA GCC GTG GGG CTG TCG GGC ACC TTC CGG GCT
ACG AAG GTC GTT CGG GCT CGG CAC CCC GAC AGC CCG TGG AAG GCC CGA

145   150   155   160   165   170   175   180   185   190
TTC CTG TCC TCT AGG CTG CAG GAT CTC TAT AGC ATC GTG CGC CGT GCT
AAG GAC AGG AGA TCC GAC GTC CTA GAG ATA TCG TAG CAC GCG GCA CGA

195   200   205   210   215   220   225   230   235   240
GAC CGG GGG TCT GTG CCC ATC GTC AAC CTG AAG GAC GAG GTG CTA TCT
CTG GCC CCC AGA CAC GGG TAG CAG TTG GAC TTC CTG CTC CAC GAT AGA

245   250   255   260   265   270   275   280   285
CCC AGC TGG GAC TCC CTG TTT TCT GGC TCC CAG GGT CAA CTG CAA CCC
GGG TCG ACC CTG AGG GAC AAA AGA CCG AGG GTC CCA GTT GAC GTT GGG

290   295   300   305   310   315   320   325   330   335
GGG GCC CGC ATC TTT TCT TTT GAC GGC AGA GAT GTC CTG AGA CAC CCA
CCC CGG GCG TAG AAA AGA AAA CTG CCG TCT CTA CAG GAC TCT GTG GGT

340   345   350   355   360   365   370   375   380
GCC TGG CCG CAG AAG AGC GTA TGG CAC GGC TCG GAC CCC AGT GGG CGG
CGG ACC GGC GTC TTC TCG CAT ACC GTG CCG AGC CTG GGG TCA CCC GCC

385   390   395   400   405   410   415   420   425   430
AGG CTG ATG GAG AGT TAC TGT GAG ACA TGG CGA ACT GAA ACT ACT GGG
TCC GAC TAC CTC TCA ATG ACA CTC TGT ACC GCT TGA CTT TGA TGA CCC

435   440   445   450   455   460   465   470   475   480
GCT ACA GGT CAG GCC TCC TCC CTG CTG TCA GGC AGG CTC CTG GAA CAG
CGA TGT CCA GTC CGG AGG AGG GAC GAC AGT CCG TCC GAG GAC CTT GTC

485   490   495   500   505   510   515   520   525
AAA GCT GCG AGC TGC CAC AAC AGC TAC ATC GTC CTG TGC ATT GAG AAT
TTT CGA CGC TCG ACG GTG TTG TCG ATG TAG CAG GAC ACG TAA CTC TTA

530   535   540   545   550   555
AGC TTC ATG ACC TCT TTC TCC AAA TAG
TCG AAG TAC TGG AGA AAG AGG TTT ATC
```

Fig. 4

ENDOSTATIN

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
MOUSE	**ENDOSTATIN**			
pET17b/ his.mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:4)	*NdeI* & *XhoI*	Prokaryotic expression, pET *(E. coli his.endo)*	MGHHHHHHHHHHHHSSGHIDDDDKH M-mendo (SEQ ID NO:14)
	5' AAC TCG AGC TAT TTG GAG AAA- GAG GT-3' (down) (SEQ ID NO:5)			
pET28a/ mendo	5'-GGC ATA TGC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:4)	*NdeI* & *NotI*	Prokaryotic expression, pET *(E. coli his.endo)*	MGSSHHHHHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5'-AAG CGG CCG CCT ATT TGG AGA-AAG AGG T-3' (down) (SEQ ID NO:6)			
pET28a/ EM-1	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		Prokaryotic expression, pET *(E. coli EM1)*	MGSSHHHHHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5' TTA GCG GCC TAC TCA ATG- CAC AGG ACG ATG TA-3' (down) (SEQ ID NO:8)			
pET28a/ EM-2	5' TTC CAT ATG CAT ACT CAT CAG- GAC TTT CAG CCA-3' (up) (SEQ ID NO:7)		Prokaryotic expression, pET *(E. coli EM2)*	MGSSHHHHHHHHHHSSGLVPRGSHM-mendo (SEQ ID NO:15)
	5' TTA GCG GCC GCC TAG TTG TGG- CAG CTC GCA GCT TTC TG-3' (down) (SEQ ID NO:9)			

Fig. 5A

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ mendo *(yeast mus endo)*	5' GGG AAT TCC ATA CTC ATC AGG- ACT TT-3' (up) (SEQ ID NO:10)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast mouse endostatin)*	EF-mendo (SEQ ID NO:16)
	5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)			
pPICZαA/ His.mendo	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast mouse his.endostatin)*	EFMGHHHHHHHHHHHHSSGHIDDDD KHM-mendo (SEQ ID NO:17)
	5' AAG CGG CCG CCT ATT TGG AGA- AAG AGG T-3' (down) (SEQ ID NO:6)			
HUMAN	**ENDOSTATIN**			
pPICZαA/ Hendo	5' TTT GAA TTC GCC CAC AGC CAC- CGC GAC TTC CAG CCG GTG CTC- CAC-3' (up) (SEQ ID NO:12)	*Eco*RI & *Not*I	Eukaryotic expression, yeast/pPICZαA *(yeast human endostatin)*	EF-hendo (SEQ ID NO:18)
	5' AAA AGC GGC CGC CTA CTT GGA- GGC AGT CAT GAA GCT GTT CTC- AAT-3' (down) (SEQ ID NO:13)			

Fig. 5B

FIG.6A

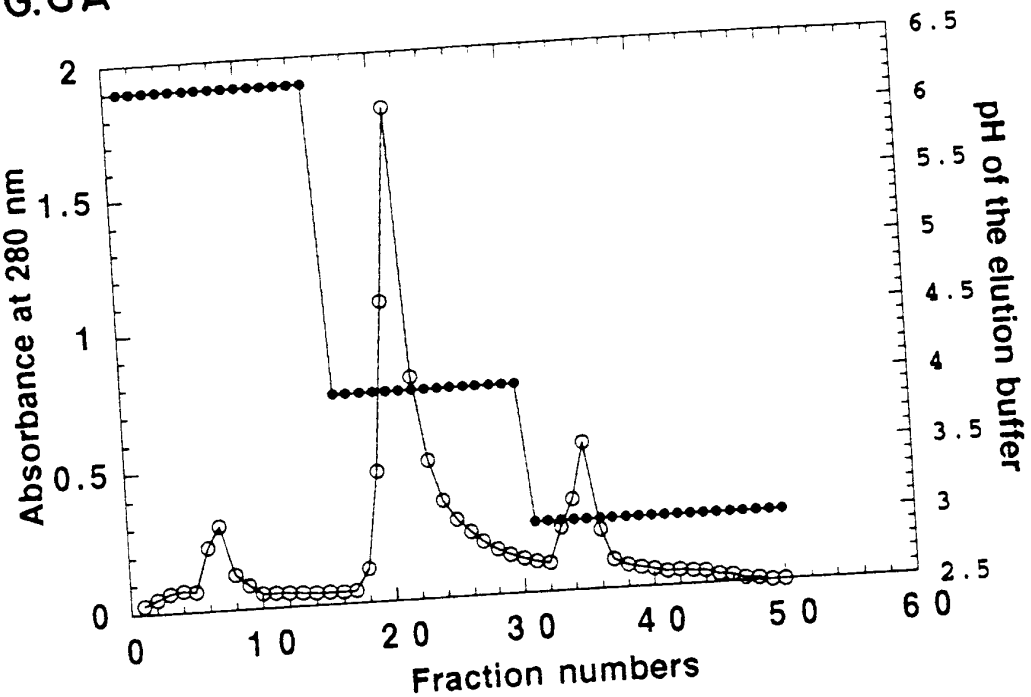

Absorbance at 280 nm
pH of the elution buffer
Fraction numbers
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
0
10
20
30
40
50
60

FIG.6B

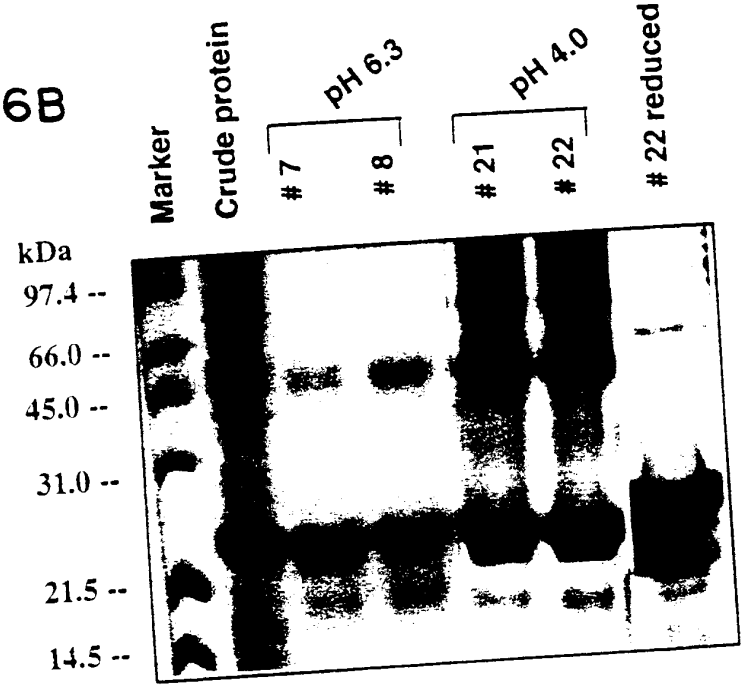

Marker
Crude protein
pH 6.3
7
8
pH 4.0
21
22
22 reduced
kDa
97.4 --
66.0 --
45.0 --
31.0 --
21.5 --
14.5 --

FIG. 7A

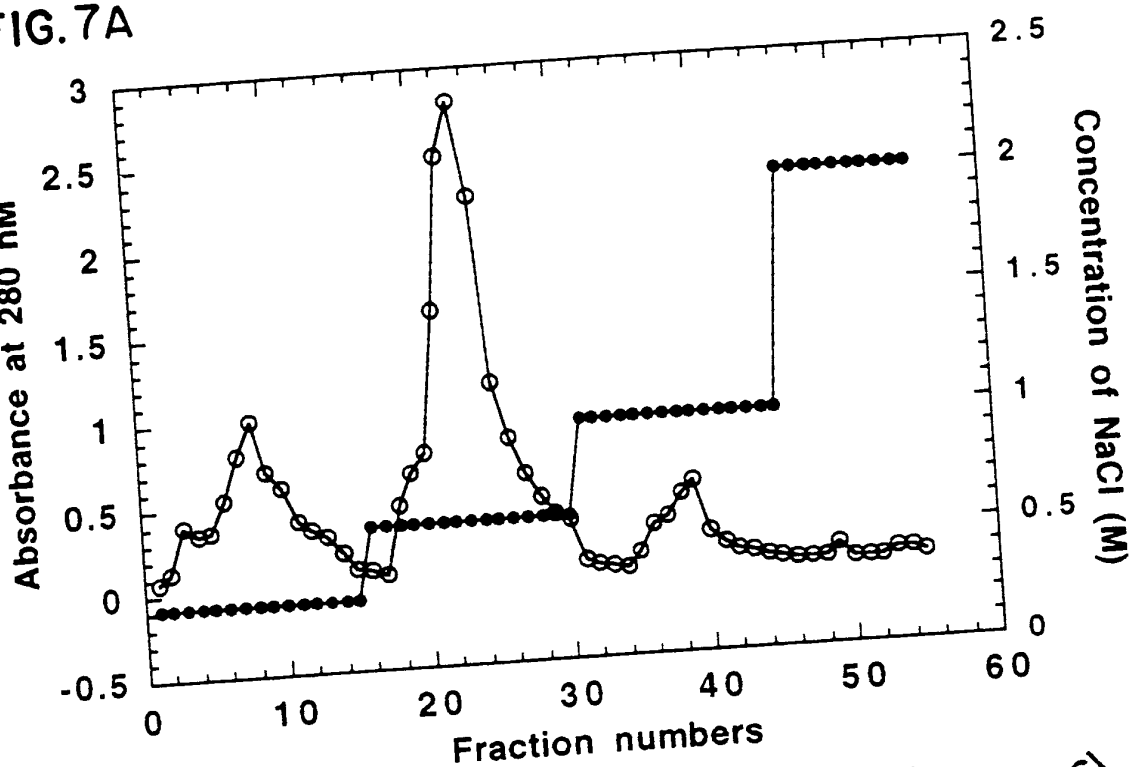

Absorbance at 280 nM
Concentration of NaCl (M)
Fraction numbers
3
2.5
2
1.5
1
0.5
0
-0.5
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60

FIG. 7B

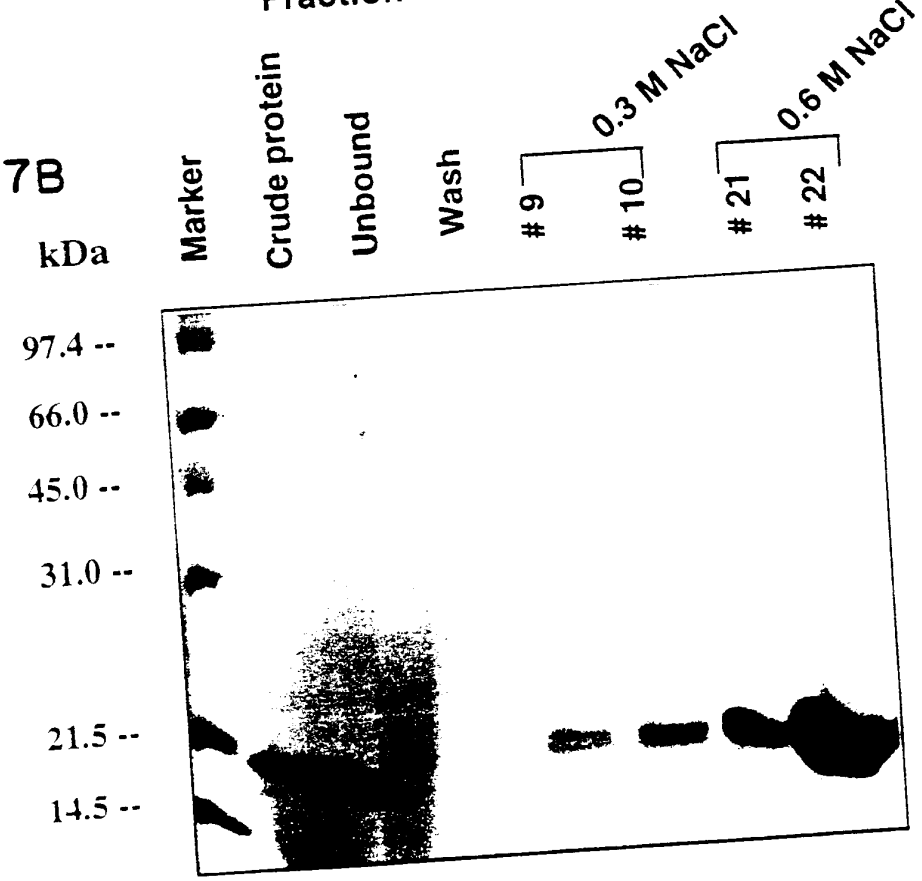

kDa
Marker
Crude protein
Unbound
Wash
0.3 M NaCl
9
10
0.6 M NaCl
21
22
97.4 --
66.0 --
45.0 --
31.0 --
21.5 --
14.5 --

FIG.8A

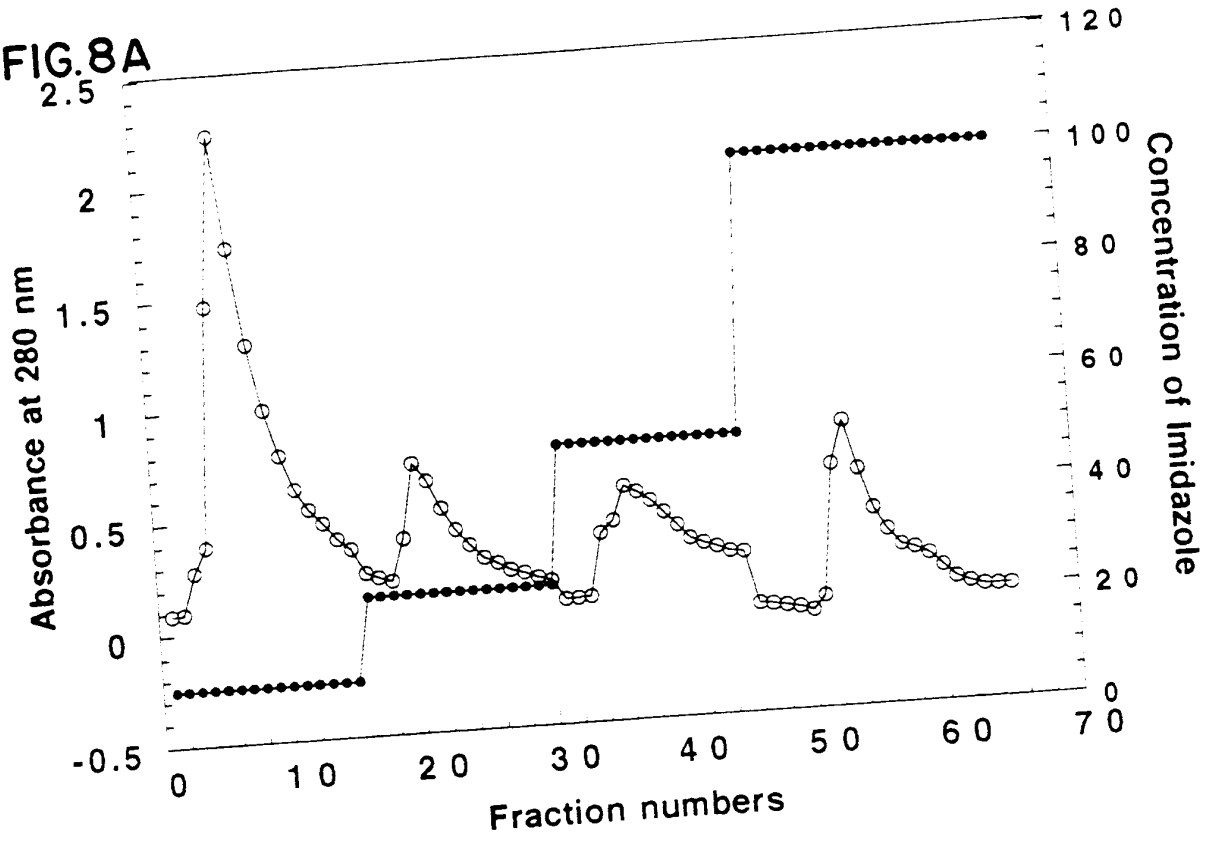
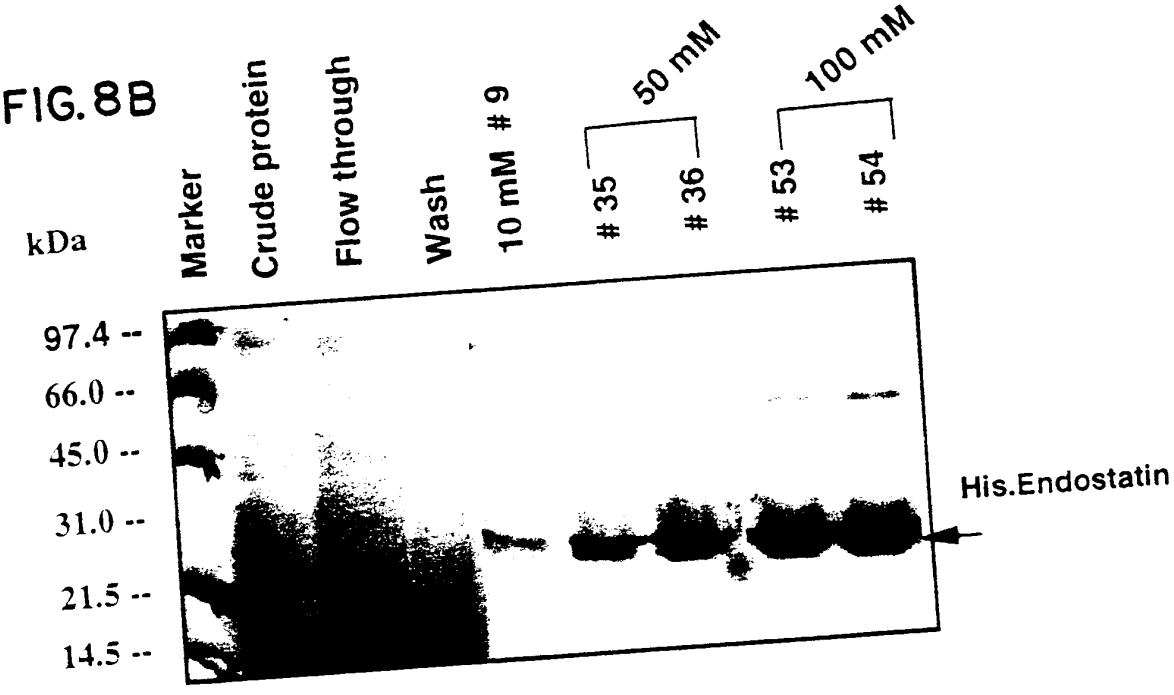

Absorbance at 280 nm
2.5
2
1.5
1
0.5
0
-0.5
0
10
20
30
40
50
60
70
Fraction numbers
120
100
80
60
40
20
0
Concentration of Imidazole

FIG.8B


kDa
Marker
Crude protein
Flow through
Wash
10 mM # 9
50 mM
35
36
100 mM
53
54
97.4 --
66.0 --
45.0 --
31.0 --
21.5 --
14.5 --
His.Endostatin

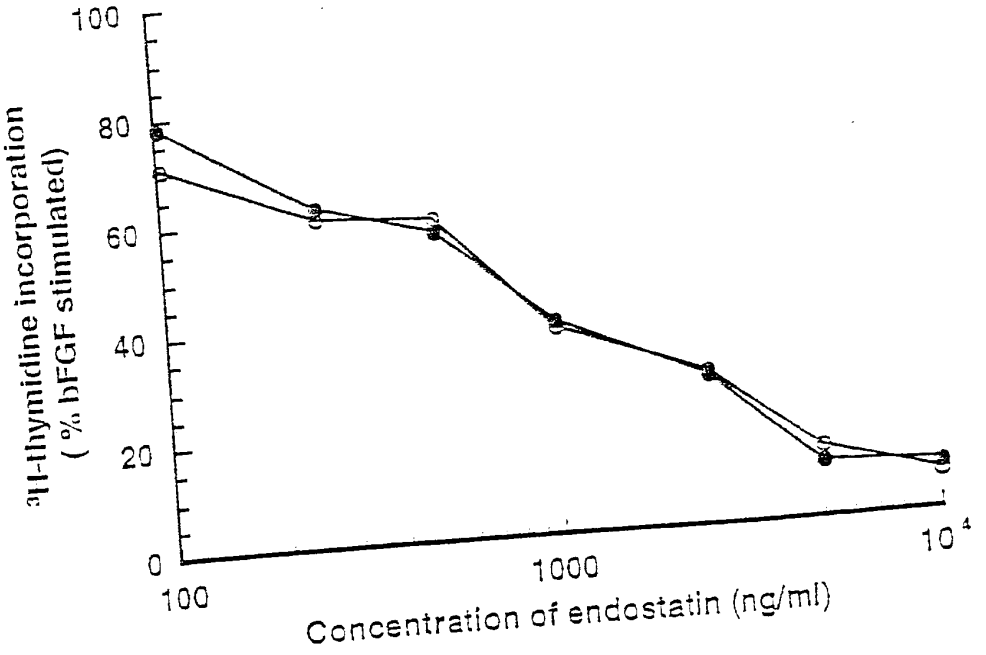

100
80
60
40
20
0
100
1000
10^4
^{3}[I-thymidine incorporation
(% bFGF stimulated)
Concentration of endostatin (ng/ml)

Fig. 9

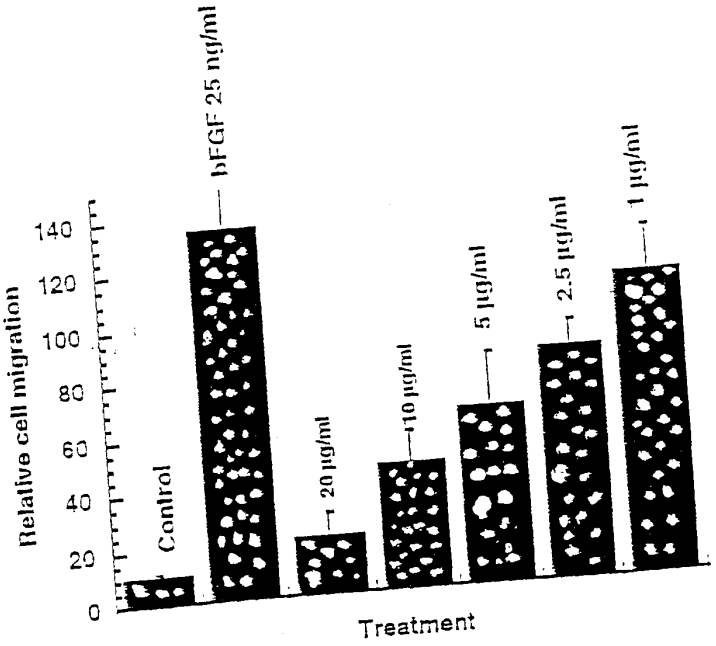

Relative cell migration
0
20
40
60
80
100
120
140
Control
bFGF 25 ng/ml
20 µg/ml
10 µg/ml
5 µg/ml
2.5 µg/ml
1 µg/ml
Treatment

Fig. 10

Fig. 11A

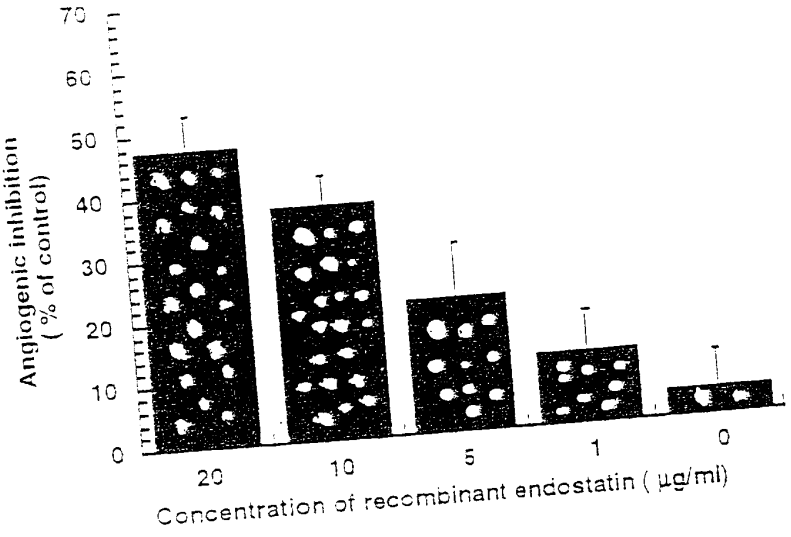
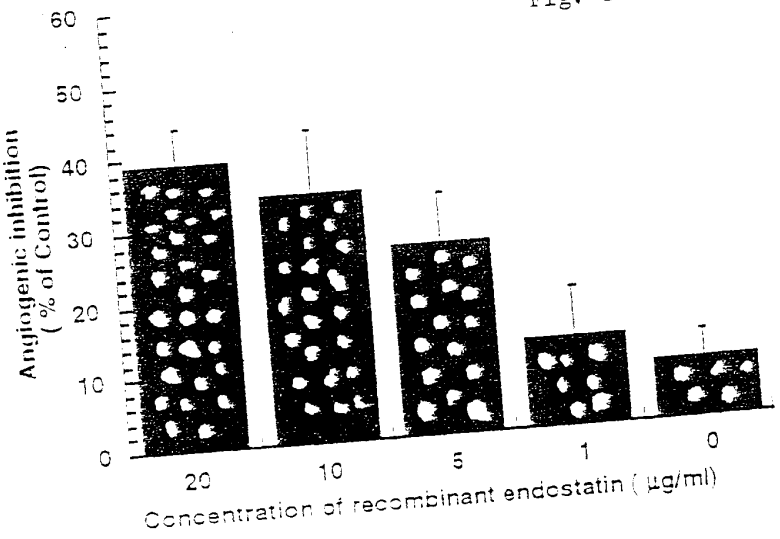


Fig. 11B



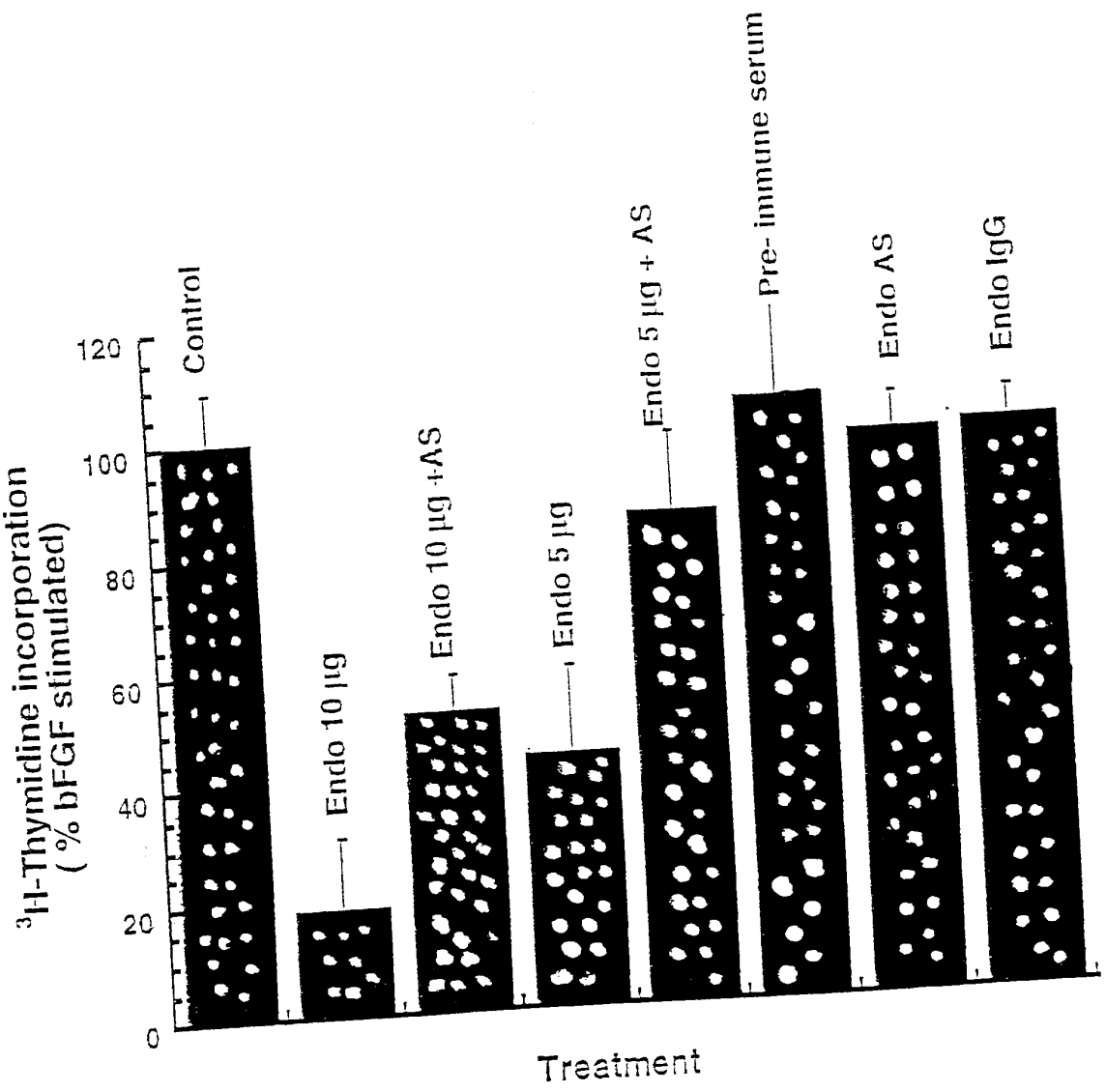

^{3}H-Thymidine incorporation
(% bFGF stimulated)
120
100
80
60
40
20
0
Control
Endo 10 µg
Endo 10 µg +AS
Endo 5 µg
Endo 5 µg + AS
Pre- immune serum
Endo AS
Endo IgG
Treatment

Fig. 12

Fig. 13A

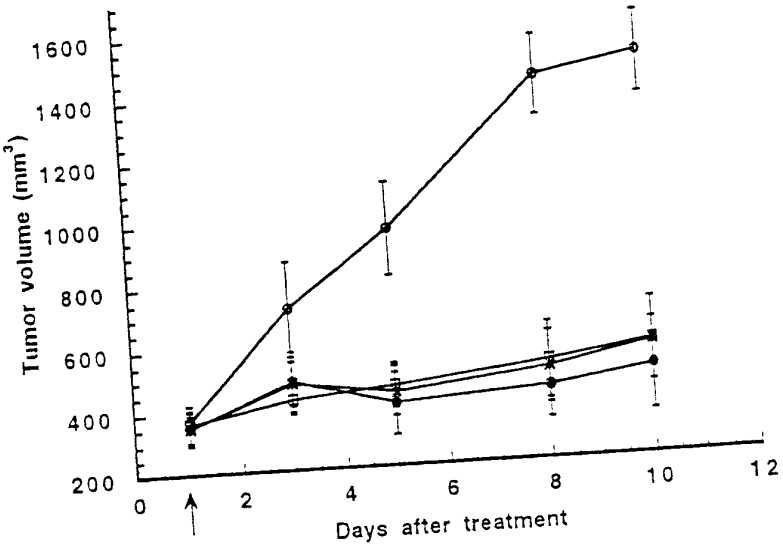


Fig. 13B

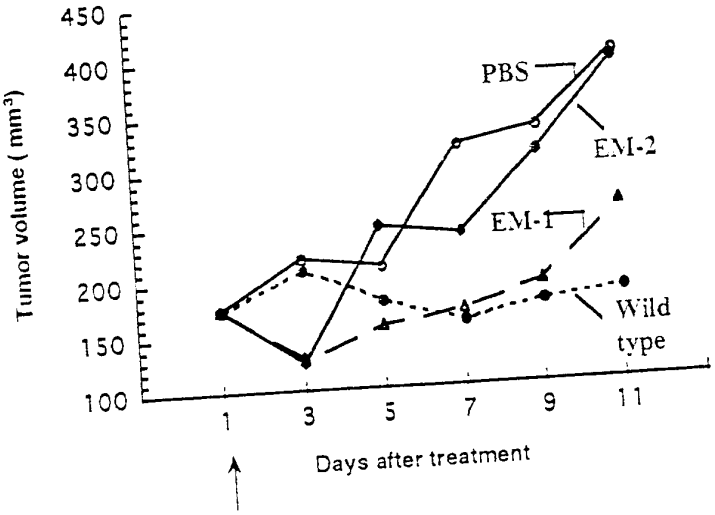


FIG.14A

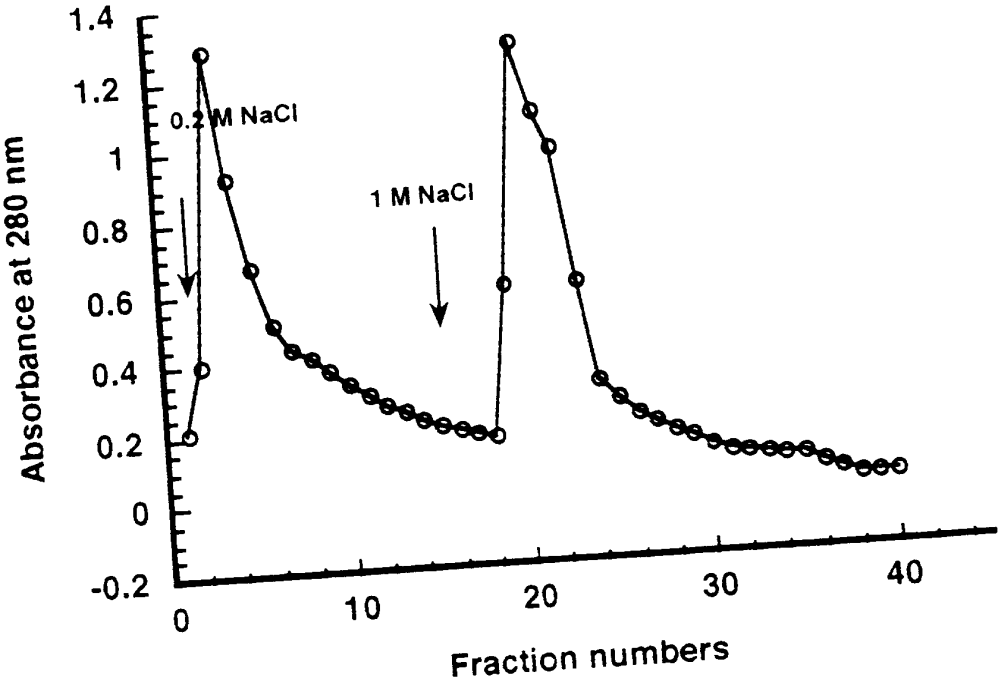


FIG.14B

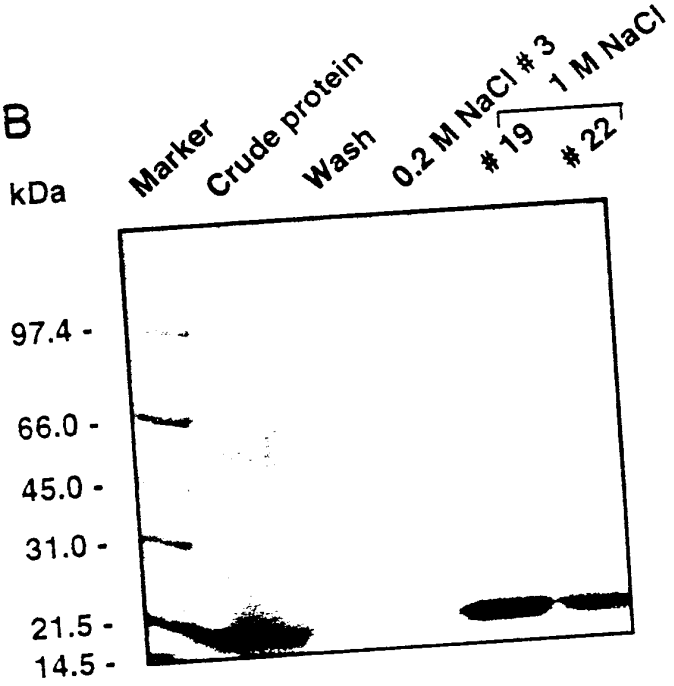


Fig. 15A

HUVE

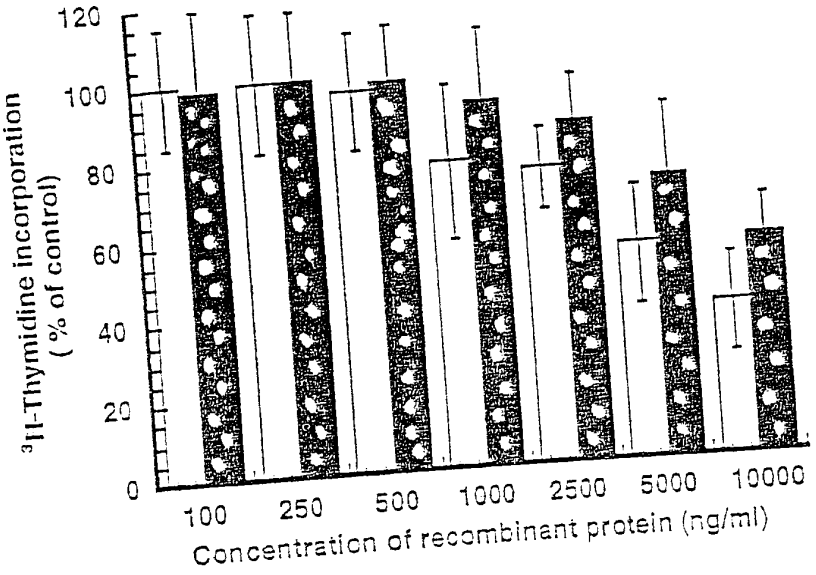


Fig. 15B

HMEV-L

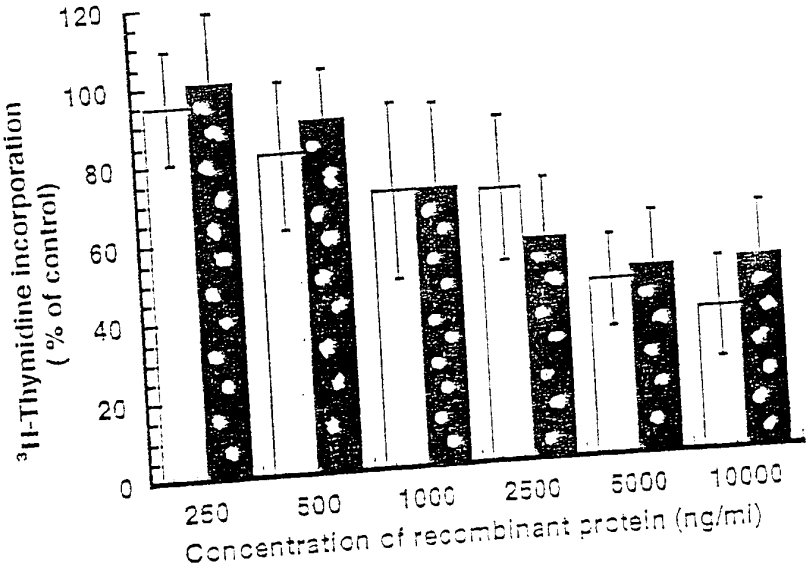


Fig. 16A

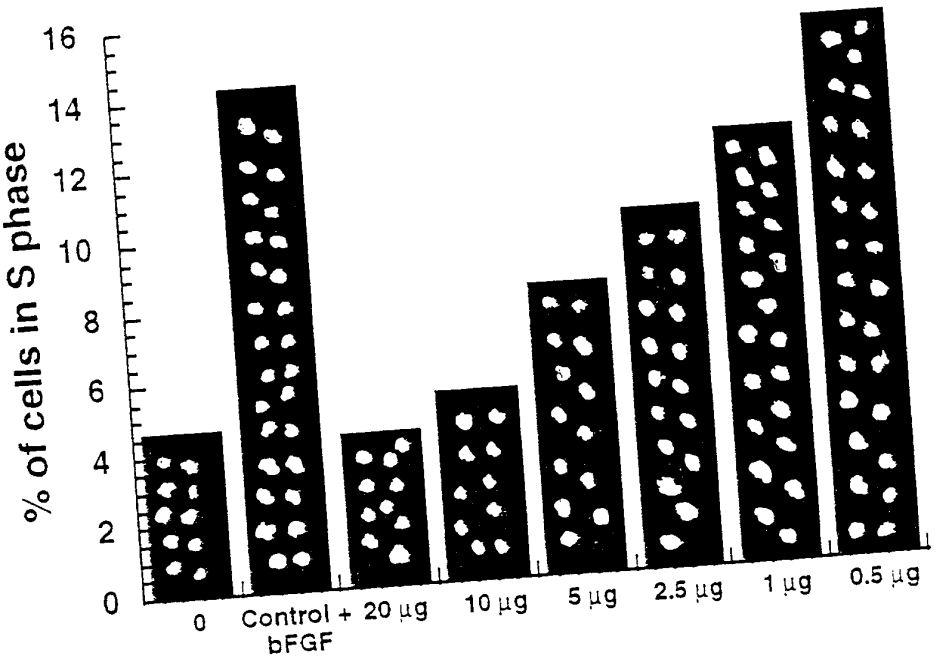


Fig. 16B

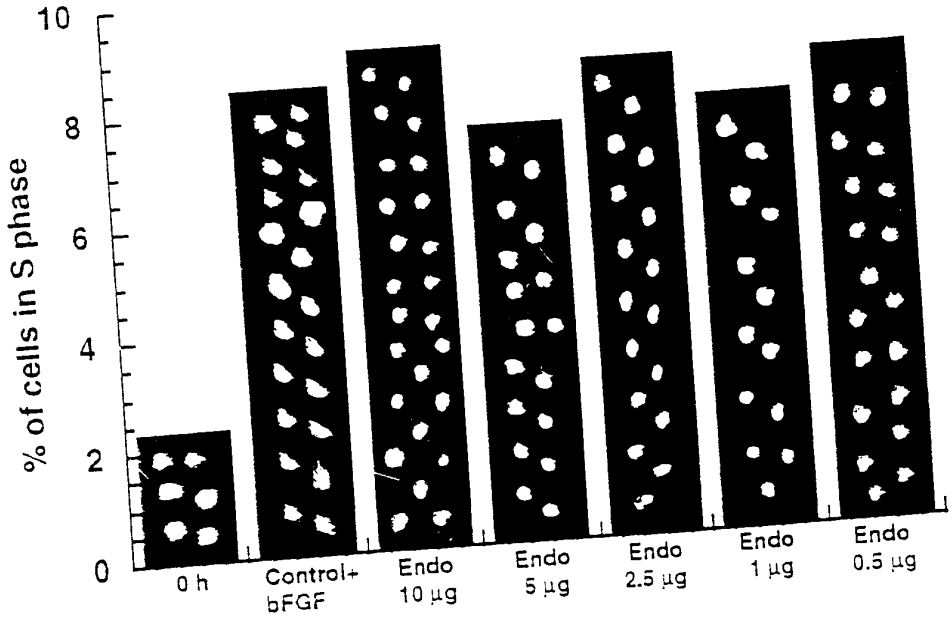


Fig. 16C

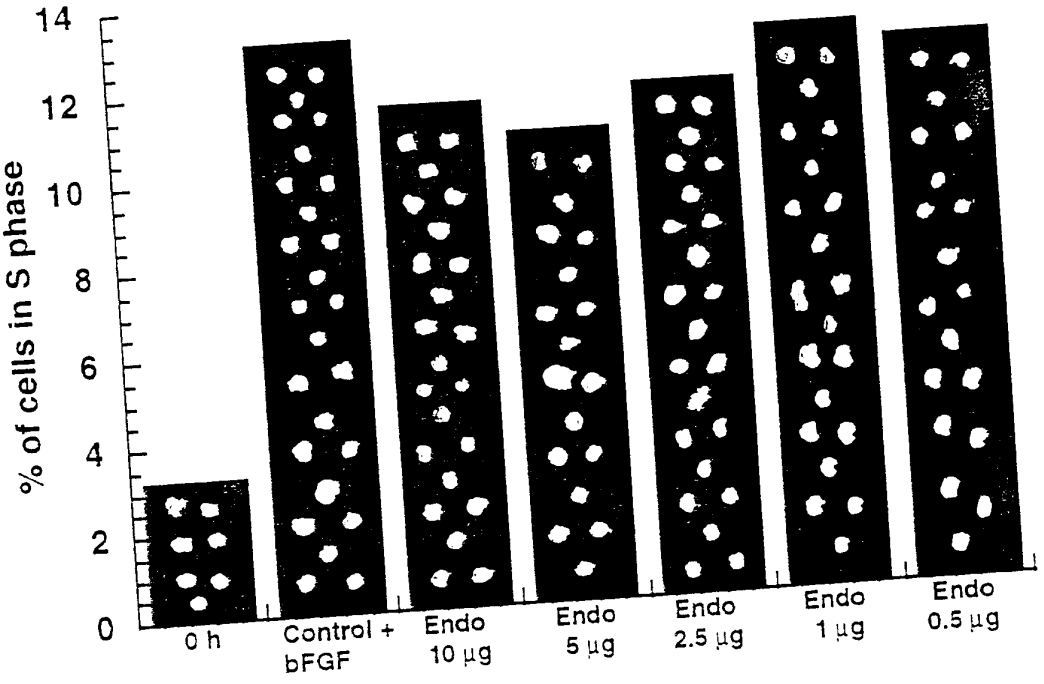
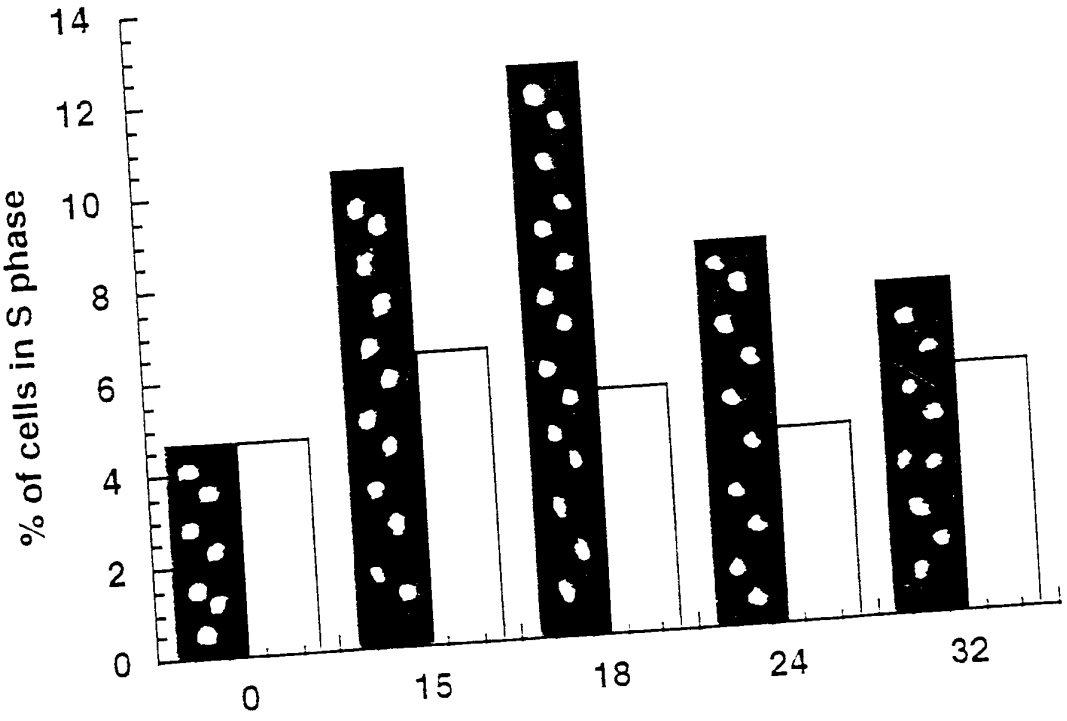


Fig. 16D



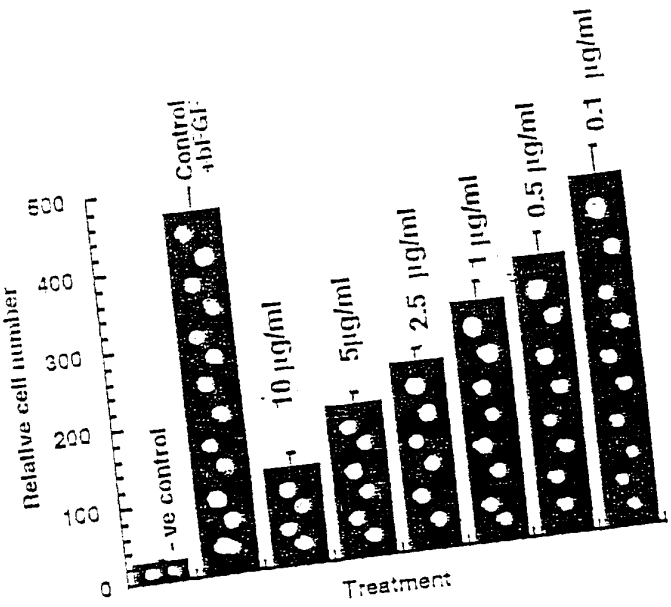

Relative cell number
0
100
200
300
400
500
- ve control
Control
+bFGF
10 µg/ml
5µg/ml
2.5 µg/ml
1 µg/ml
0.5 µg/ml
0.1 µg/ml
Treatment

Fig. 17

FIG.18A Purification of Angiostatin(mouse): Lysine Sepharose 4B column

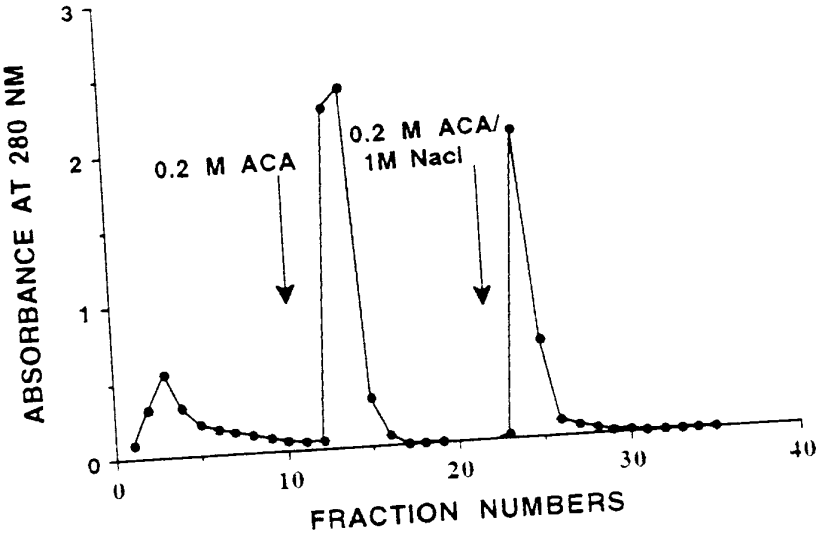
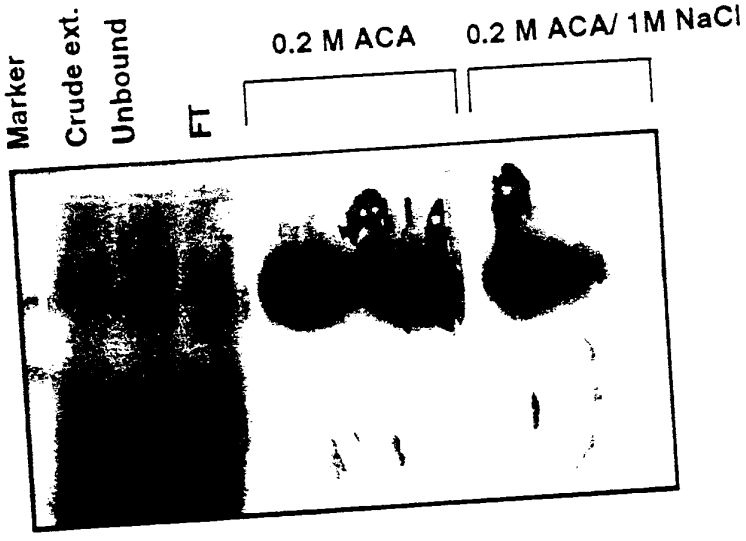


FIG.18B



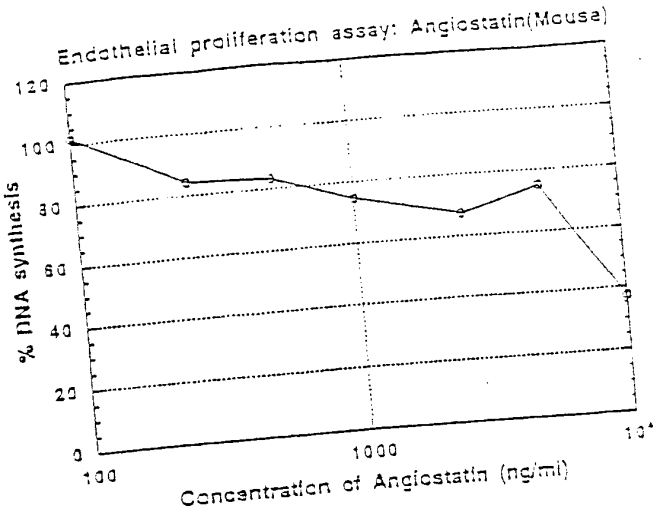

Endothelial proliferation assay: Angiostatin(Mouse)
% DNA synthesis
120
100
80
60
40
20
0
100
1000
Concentration of Angiostatin (ng/ml)

Fig. 19

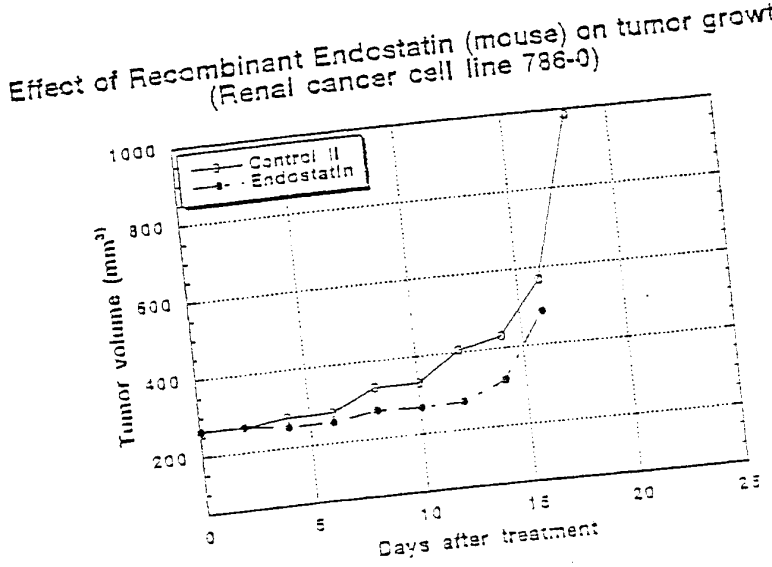
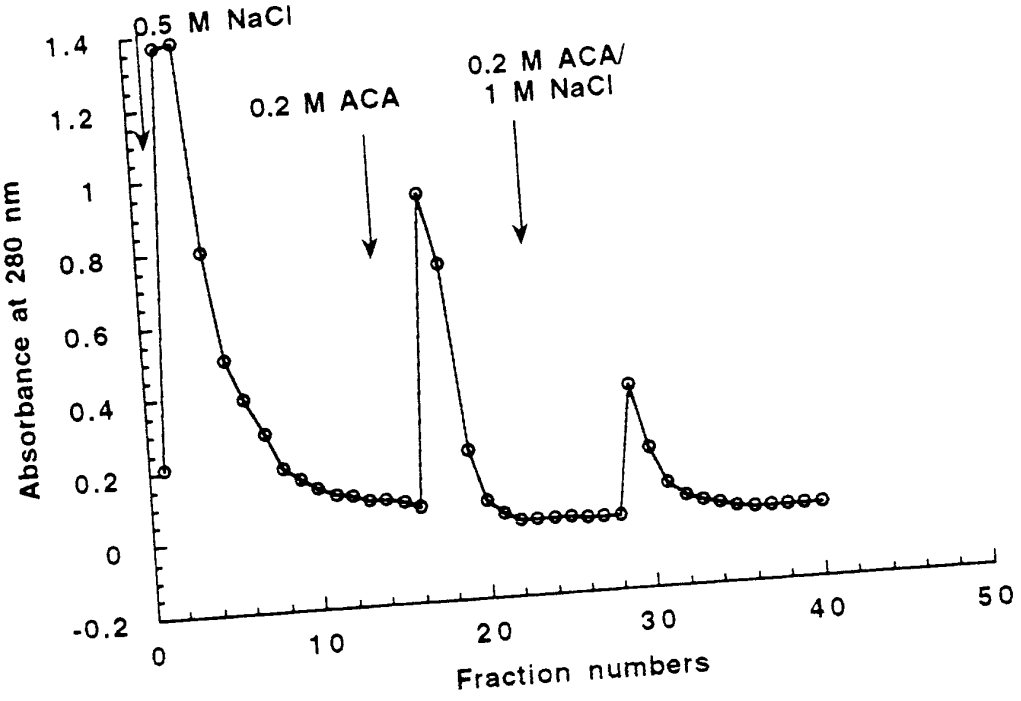

Effect of Recombinant Endostatin (mouse) on tumor growth
(Renal cancer cell line 786-0)
Control II
Endostatin
Tumor volume (mm3)
1000
800
600
400
200
0
5
10
15
20
25
Days after treatment

Fig. 20

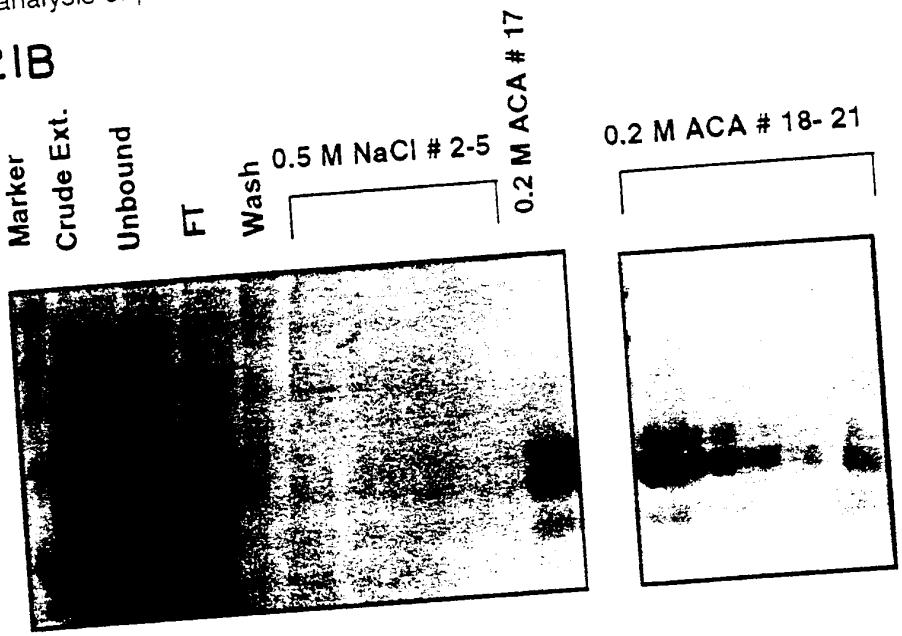
FIG.21A

Purification of Angiostatin (Human):Lysine Sepharose column.



SDS-PAGE analysis of purified recombinant Angiostatin(Human): Lysine Sepharose column

FIG.21B



Sequence Range: 1-546
Upstream primers for Restin and Apomigren are underlined, downstream primer for both is double underlined. Primer nuclectides not in the Restin sequence are shown in lower case.

```
ttt ttt gaa ttg->
         5    10    15    20    25    30    35    40    45
->ATT TCA AGT GCC AAT TAT GAG AAG CCT GCT CTG CAT TTG GCT GCT CTG
  TAA AGT TCA CGG TTA ATA CTC TTC GGA CGA GAC GTA AAC CGA CGA GAC

  50    55    60    65    70    75    80    85    90    95
  AAC ATG CCA TTT TCT GGG GAC ATT CGA GCT GAT TTT CAG TGC TTC AAG
  TTG TAC GGT AAA AGA CCC CTG TAA GCT CGA CTA AAA GTC ACG AAG TTC

   100   105   110   115   120   125   130   135   140
   CAG GCC AGA GCT GCA GGA CTG TTG TCC ACC TAC CGA GCA TTC TTA TCT
   GTC CGG TCT CGA CGT CCT GAC AAC AGG TGG ATG GCT CGT AAG AAT AGA

 145   150   155   160   165   170   175   180   185   190
   TCC CAT TTG CAA GAT CTG TCC ACC ATT GTG AGG AAA GCA GAG AGA TAC
   AGG GTA AAC GTT CTA GAC AGG TGG TAA CAC TCC TTT CGT CTC TCT ATG

   195   200   205   210   215   220   225   230   235   240
   AGC CTT CCC ATA GTG AAC CTC AAG GGC CAA GTA CTT TTT AAT AAT TGG
   TCG GAA GGG TAT CAC TTG GAG TTC CCG GTT CAT GAA AAA TTA TTA ACC

     245   250   255   260   265   270   275   280   285
   GAC TCA ATT TTT TCT GGC CAC GGA GGT CAG TTC AAT ATG CAT ATT CCA
   CTG AGT TAA AAA AGA CCG GTG CCT CCA GTC AAG TTA TAC GTA TAA GGT

   ttc cat atg->
   290   295   300   305   310   315   320   325   330   335
->ATA TAC TCC TTT GAT GGT CGA GAC ATA ATG ACA GAT CCT TCT TGG CCC
  TAT ATG AGG AAA CTA CCA GCT CTG TAT TAC TGT CTA GGA AGA ACC GGG

    340   345   350   355   360   365   370   375   380
   CAG AAA GTC ATT TGG CAT GGC TCC AGC CCC CAT GGC GTC CGC CTT GTG
   GTC TTT CAG TAA ACC GTA CCG AGG TCG GGG GTA CCG CAG GCG GAA CAC

 385   390   395   400   405   410   415   420   425   430
   GAT AAC TAC TGT GAA GCA TGG CGA ACC GCG GAC ACA GCG GTC ACG GGA
   CTA TTG ATG ACA CTT CGT ACC GCT TGG CGC CTG TGT CGC CAG TGC CCT

   435   440   445   450   455   460   465   470   475   480
   CTT GCC TCC CCG CTG AGC ACG GGG AAG ATT CTG GAC CAG AAA GCA TAC
   GAA CGG AGG GGC GAC TCG TGC CCC TTC TAA GAC CTG GTC TTT CGT ATG

     485   490   495   500   505   510   515   520   525
   AGC TGT GCT AAT CGG CTA ATT GTC CTA TGT ATC GAA AAC AGT TTC ATG
   TCG ACA CGA TTA GCC GAT TAA CAG GAT ACA TAG CTT TTG TCA AAG TAC

   530   535   540   545
   ACA GAC GCT AGG AAG TAA
   TGT CTG CGA TCC TTC ATT CGC CGC CGT AAG AA
```

Figure 22

Flow Chart: Cloning of Restin (ColXV) into Pichia Expression System

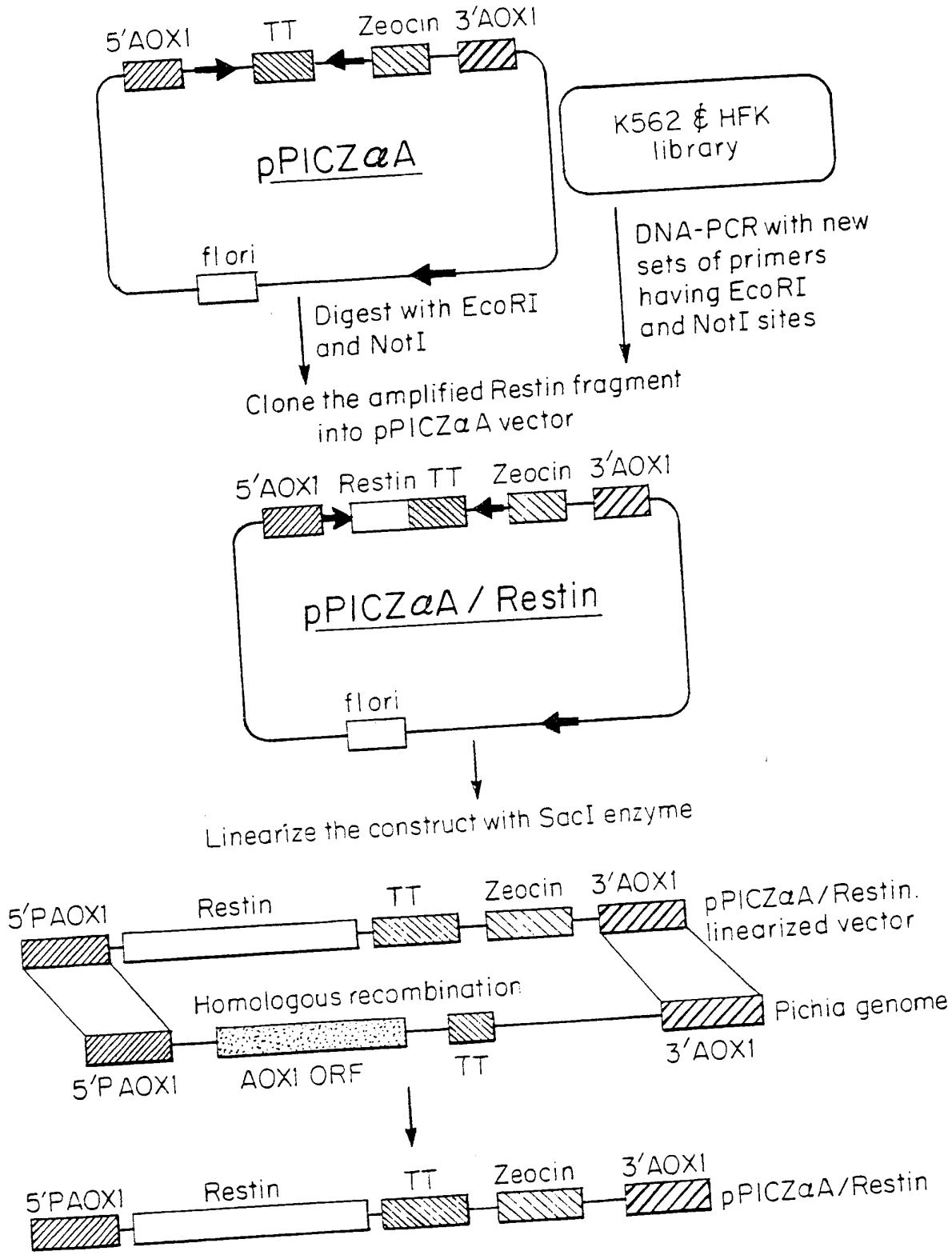


Fig. 24

Sequence Range: 1 to 181

```
  5   10  15  20  25  30  35  40  45
ISS ANY EKP ALH LAA LNM PFS GDI RAD FQC FKQ ARA AGL LST YRA FLS

50   55  60  65  70  75  80  85  90  95
SHL QDL STI VRK AER YSL PIV NLK GQV LFN NWD SIF SGH GGQ FNM HIP

 100 105 110 115 120 125 130 135 140
IYS FDG RDI MTD PSW PQK VIW HGS SPH GVR LVD NYC EAW RTA DTA VTG

145  150  155  160  165  170  175  180
LAS PLS TGK ILD QKA YSC ANR LIV LCI ENS FMT DAR K
```

Fig. 23

HUMAN RESTIN

Construct Name	Primer Sequence	Cloning Sites	Vector	Protein Sequence
pPICZαA/ Restin	5' TTT TTT GAA TTC ATT TCA AGT- GCC AAT TAT GAG AAG CCT GCT- CTG CAT- TTG-3' (up) (SEQ ID NO:21)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human restin)*	EF-restin (SEQ ID NO:27)
	5' AAG AAT GCG GCC GCT TAC TTC- CTA GCG TCT GTC ATG AAA CTG- TTT TCG AT-3' (down) (SEQ ID NO:22)			
pPICZαA/ His.Restin	5' AAT TCC ATC ACC ATC ACC ATC- ACG- 3' (up) (SEQ ID NO:23)	*Eco*RI (oligo insertion)	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human his.restin)*	EFHHHHHHH-restin (SEQ ID NO:28)
	5' AAT TCG TGA TGG TGA TGG TGA- TGG-3' (down) (SEQ ID NO:24)			
pET28a/ apomigren	5' TTC CAT ATG ATA TAC TCC TTT- GAT GGT CGA GAC ATA ATG ACA-3' (up) (SEQ ID NO:25)	*Nde*I & *Not*I	Prokaryotic, pET system *(E.coli human apomigren)*	MGSSHHHHHHSSGLVPRGSHM-apo migren (SEQ ID NO:29)
	5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)			
pPICZαA/ apomigren	5' AAG AAT TCC ATC ATC ATC ATC- ATC ACA GCA GC-3' (up) (SEQ ID NO:11)	*Eco*RI & *Not*I	Eukaryotic (Yeast), Pichia, pPICZαA *(yeast human apomigren)*	EFMGSSHHHHHHSSGLVPRGSHM-apomigren (SEQ ID NO:30)
	5' AAT GCG GCC GCT TAC TTC CTA- GCG TCT GTC ATG AAA CTG TTT- TCG AT-3' (down) (SEQ ID NO:26)			

Fig. 25

No. of cells migrated
0
100
200
300
400
500
Control -bFGF
Control +bFGF
3
6.25
12
25
50
Concentration of reslin (μg/ml)

Fig. 26

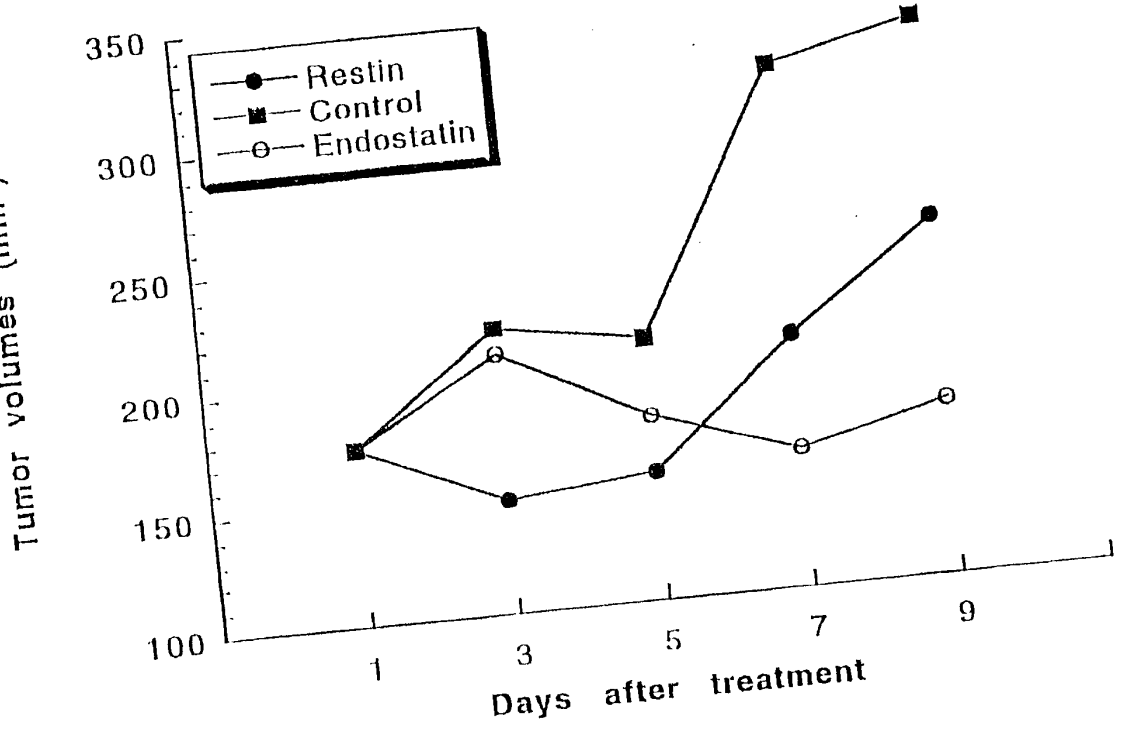

Restin
Control
Endostatin
Tumor volumes (mm^3)
350
300
250
200
150
100
1
3
5
7
9
Days after treatment

Fig. 27